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13. ABSTRACT ***(Maximum 200 Words)***

The immunotherapeutic potential of the antibody-cytokine fusion proteins has been investigated as an alternative means of the therapy for refractory breast cancer. These novel molecules contain the antibody portion recognizing the tumor associated antigen and is covalently linked to a potent immune stimulator. Experiments were performed to elucidate the mechanisms of targeting tumor cells for destruction and mechanisms of stimulation immune effector cells by these molecules, to ultimately translate these findings to clinical application. Initially new delivery approach of fusion protein was studied, based on particle-mediated gene transfer (PMGT), and most effective expression vectors were developed. Animal model for tumor immunotherapy was established, using cancer cell lines engrafted to syngeneic animals. After gene delivery detectable expression of fusion protein was achieved. Despite of lack of therapeutic effect of this approach in animal model, substantial progress has been made in studies with fusion protein used as a protein reagent. *In vitro* was demonstrated that fusion protein can specifically bind to cancer cells and deliver IL-2 to their surface. It can activate IL-2 responsive cells and induce tumor specific ADCC. Sensitive assays for detection of fusion protein and its components in animal and patient's serum were developed. It was shown that after administration this molecule is stable for several hours in animal and human serum (confirmed also on clinical samples from separate trial of similar fusion protein of different specificity), retains its functional activity and induces immune activation. These findings will provide a model for bringing novel immunocytokines to the clinic to improve effectiveness of human cancer treatment.

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INTRODUCTION

New directions in alternative treatment of breast cancer involve the use of monoclonal antibodies directed against breast tumor associated antigens. Several types of these antigens have been recently identified. These include the epidermal growth factor related HER-2/neu proto-oncogene and cell surface antigens such as MH-99 glycoprotein, EpCAM and TAG-72 from the mucin family. These molecules have been examined as a targets for disease detection, radioimaging and immunotherapy. Numerous studies have demonstrated the value of antibodies against these antigens in prognostic and diagnostic applications, and have shown potential utility in animal models, *in vitro* settings, and in early clinical experiments. Also cytokines have been actively considered as one of the most promising approaches for treatment of advanced cancers, including metastatic breast tumor (1), especially because of the possibility of eradication of the primary tumor site as well as metastases, through the activation of anti-tumor immunity mechanisms.Using recombinant DNA technology, it has been possible to extend the utility of these molecules by altering their configuration to better suit specific needs (2).

Extensive investigation is taking place in the area of the use of antibodies to serve as delivery vehicles for immune stimulating agents that can target tumor cells for destruction directly or by attracting and stimulating resident immune effector cells. We have been interested in examining the immunotherapeutic effectiveness of antibody-cytokine fusion proteins. In these molecules the antibody portion recognizes the tumor associated antigen, and is covalently linked to a potent immune stimulator (3).

We were investigating the potential advantages of delivering this kind of fusion protein by a novel gene therapy mechanism, using particle mediated gene transfer (PMGT) to transiently transfect a fusion protein gene into murine epithelial cells at the site of delivery (4).

The initial goal of our approach was to provide effective concentrations of both tumor reactive chimeric antibody and IL-2 in the microenvironment of metastatic breast lesions. Our studies initially addressed the question of whether immunotherapeutic levels of an antibody-cytokine fusion protein can be achieved by gene therapy, i.e. by delivering DNA encoding the fusion protein to tumor bearing animals. We utilized the transfection procedure known as particle mediated gene transfer, in which DNA-coated gold particles are accelerated by a compressed gas discharge into cells or organisms (5). Previously we had shown that substantial skin and detectable serum levels of the CC49-IL2 fusion protein were achieved following PMGT into murine epidermis (4). However, expression levels were not as high as those obtainable by parenteral administration of the protein itself. Data from other studies have documented that *in vivo* delivery of an antibody-IL2 FP provides more efficient tumor destruction and more significant survival benefit in tumor-bearing mice than treatment with equivalent doses of IL-2 alone, mAb alone, or the combination of antibody and IL-2 given as separate molecules at equivalent doses to that provided by the FP (6). As little as 1 μg of FP injected intraperitoneally was sufficient to provide a significant therapeutic benefit. This corresponds to only about 1% of the maximal tolerated dose of IL-2 in mice, suggesting the highly bioactive nature of this type of fusion protein. Therefore we were very enthusiastic about testing this new approach.

BODY :

It was important to determine whether we could augment expression levels of the CC49-IL2 fusion protein (FP) beyond those seen with the current vector used, since expression is a critical parameter in any gene therapy model. Therefore we generated and analyzed a series of novel expression vectors. The vector we used for our initial studies on the CC49-IL2 fusion protein was derived from the commonly used pLNCX plasmid (7), and was denoted as pLNC-CCIL. This vector is from a family of recombinant replication defective retroviruses, and utilizes the eukaryotic-selectable marker neomycin. The gene of interest was cloned downstream of the CMV promoter, which is active in most mammalian cell types (8). As described in previous publications, we have used the pLNCX based expression system to generate information on *in vitro* and *in vivo* produced protein (4).

The B16 murine melanoma cell line was used for the *in vitro* expression analyses, since these cells are effective recipients for particle delivery, and efficiently use the CMV promoter. After PGMT, medium was added and the cells were incubated for 24 hours prior to assaying the medium for secreted FP expression.

To analyze expression *in vivo*, BALB/C mice were used, and particles were delivered at 350 psi to the exposed skin. 24 hours following delivery, the animals were sacrificed. Skin biopsies of the recipient epidermal area was subjected to extraction procedure and obtained supernatant was analyzed.

In both the *in vivo* and *in vitro* experiments, fusion protein levels were quantitated using ELISA specific for human IL-2. The assay was sensitve to approximately 3 pg/ml of IL-2, corresponding to a level of 15 pg/ml of the fusion protein. The expression studies demonstrated that the pLNCX derived vector pLNC-CCIL generated the highest levels of CC49-IL2 fusion protein both *in vitro* and *in vivo*. Therefore, we decided to carry out further experiments using this vector.

Prior to the development of an *in vivo* immunotherapy model utilizing CC49-IL2 FP, we examined the ability of the protein to localize specifically to tumor sites that expressed the target antigen. This was done by the use of PMGT to deliver the gene encoding the CC49-IL2 FP into mouse epidermis overlying a TAG-72+ tumor. Tissue samples were obtained at 24 and (from different animals) at 96 hours. The expectation was that at the 96 hour time point more of the FP would be retained in the antigen positive site (the LS174T tumor) than in the other 2 antigen negative sites. Unfortunately, the obtained data showed no evidence of fusion protein retention at the LS174T tumor compared to what was seen at the other 2 sites.

We also attempted to evaluate FP produced by gene delivery to exert anti-tumor effects in conjunction with adoptively transferred human effector cells. We used the approach with adoptively transferred cells based on data generated in previous experiments. We chose to develop a syngeneic model in which TAG-72+ murine tumors were grown in immunocompetent animals sharing the genetic background of the tumor.

Despite the fact that no significant differences were noted in these experiments, the kinetics of the response indicated a subtle slowdown in tumor growth in the experimental group (transfected tumor exposed to the CC49-IL2 expression plasmid). However, this decreased growth rate seen at days 6 to 10 was not maintained over the course of the experiment.

As summarized above, the level of gene expression (i.e. local and systemic CC49-IL2 protein levels) was insufficient for *in vivo* anti-tumor efficacy. Furthermore, a separate fusion protein (KS-IL2, see below), which is highly reactive with breast cancer, and at this point was available to us, as a purified protein reagent, in sufficient quantities to pursue the pre-clinical *in vitro* and *in vivo* studies we initially proposed with CC49-IL2. The KS1/4 murine monoclonal antibody (mAb), generated initially by R.Reisfeld and colleagues is reactive with a membrane molecule found to be heavily expressed on human cancer cells including breast, lung, colon and prostate (9). The MH-99 antigen recognized by this antibody is EP-CAM molecule, strongly present on adenocarcinoma cells, and with low level of expression on certain normal epithelial cell types including mucin rich cells of the GI system. (10) Preliminary clinical testing of the KS1/4 antibody in patients with measurable

disease showed relatively little toxicity or antitumor effect (11). We intended to test preclinical strategies utilizing a molecular derivative of the KS1/4 antibody, designed to provide enhanced clinical efficacy. Two major molecular modifications within the molecule were created: first - grafting the murine hypervariable regions (responsible for antigen binding) to a human IgG framework to achieve a "humanized" KS1/4 antibody able to mediate ADCC. Secondly, linking human interleukin-2 by genetic construction to the carboxy terminus of each heavy chain of this humanized antibody, completed the design of the fusion protein, designated huKS-IL2.(12) The principal action of this new molecule is the activation of the immune system through IL-2 and Fc receptors, localized to tumor cells by the fusion protein's antibody component. This fusion protein has been recently shown to be able to mediate potent antitumor effects against established lung and liver metastases in syngeneic mice bearing tumors which express the KSA (MH-99) antigen.

This syngeneic tumor model created for evaluation of the humanized KS-IL2 fusion protein was based on the CT26 colon carcinoma, which originated in BALB/C mice. This is a weakly immunogenic tumor that can induce a protective immune response following appropriate syngeneic immunization.(13) The variant of these tumor cells, CT-26/KSA has been achieved after transfection of parental cells with the human KSA gene, and expresses the KSA molecule on the cell surface, allowing the recognition by the KS1/4 antibody. (14) Following intravenous or intrasplenic injection of these cells into animals, metastases arise in the liver as well as lungs and are rapidly fatal. Animals treated with the KS1/4 antibody together with IL-2 show a striking decrease in the number and size of their metastases; however, considerable metastatic disease remains. In contrast, daily intravenous injections of the hu KS-IL2 fusion protein for 7 days causes complete elimination of all detectable lung and liver metastases. (12) Animals that are long term survivors following this fusion protein treatment can be rechallenged with the parental CT26-KSA tumor cells and show rapid tumor rejection *in vivo.*

As this tumor associated antigen is present on breast adenocarcinoma cells as well, we attempted to utilize the animal model described above to elucidate pharmacokinetics of huKS-IL2 fusion protein and assess the potential of induction of neutralizing antibodies against fusion protein in injected animals. Our ultimate goal was to determine how this molecule is likely to be most effective in treating clinical cancer and which immune mechanisms are likely involved in this process.

In this experimental system described above we were able to evaluate by flow cytometry if the huKS-IL2 fusion protein specifically targets EP-CAM positive tumor. Indirect staining of the T47D breast tumor cell line and LS174 colon tumor cell line after incubation with fusion protein showed that the huKS-IL2 fusion protein binds to the tumor cells, bringing the IL-2 component of the molecule to the cell surface in a form recognized by anti-human IL-2 antibody. This experiment proved that the fusion protein can act as a delivery vehicle of cytokine to the tumor site.

To assess the ability of huKS-IL2 fusion protein to stimulate proliferation of IL-2 dependent cells *in vitro,* we tested this molecule as a stimulus for the TF-1β cell line, created previously in the lab, as well as PHA stimulated PBMCs from a healthy donor. Fusion protein huKS-IL2 induced comparable proliferation to soluble IL-2 of human cells expressing intermediate or high affinity IL-2 receptor complexes. This experiment proved that the IL-2 component of huKS-IL2 fusion protein retains its functional activity within the fusion protein molecule.

We have as well analysed the specificity of fusion protein in mediating tumor cell destruction with freshly obtained PBMCs from healthy donors, used as effector cells in a 4 hour chromium release assay with T47D human breast carcinoma and LS174 human colon carcinoma targets. The results indicated that huKS-IL2 fusion protein specifically binds to the T47D and LS174 tumor targets and interacts with the effector cells to facilitate ADCC. We also proved that huKS-IL2 fusion protein induces striking ADCC, in comparison to the KS1/4 antibody used with soluble IL-2 as separate molecules present in the *in vitro* system, and that the IL-2 receptor complex is involved in the boosted killing of target cells.

We also attempted to clarify the role of MHC class I expressed on the surface of tumor cells and NK cell involvement in KS-IL2 fusion protein induced killing *in vitro.* Cytotoxicity assays

performed in the presence of FP, using naive murine splenocytes as effectors and CT21.6 cells as targets, showed that killing of tumor cells occurs in a FP dose-dependent manner. Tumor cell line, CT26-Ep which express normal levels of MHC class I was killed at much lower level than the low class I expressor CT21.6. Upregulation of MHC class I on CT26.1 by in vitro IFN-γ treatment decreased the killing. This was associated with the level of induced MHC class I molecules on the surface of these tumor cells. These results suggest that the in vitro killing of CT21.6 tumor cells mediated via KS-IL2 fusion protein involves NK cells and this activity can be inhibited by upregulation of MHC class I expression and also suggests the potential involvement of the killer inhibitory receptors on NK cells in this process (16,17).

For all these *in vitro* as well *in vivo* studies we established very sensitive and specific enzyme-linked immunosorbent assay (ELISA) systems that allowed the accurate detection of intact fusion proteins, by requiring simultaneous recognition of the IL-2 and immunoglobulin components of the fusion protein molecule. (15) The huKS-IL2 fusion protein can be easily detected by the IL-2/IgG1 ELISA (which detects the intact fusion protein molecule), by the IgG1 ELISA (which detects the immunoglobulin component of the fusion protein) and by the IL-2 ELISA (which detects the cytokine component of the fusion protein).

It was anticipated that a strong mouse anti-humanized antibody response will arise in conventional mice treated with huKS-IL2 fusion protein. To evaluate this, a group of mice was analyzed during the treatment (15 μg/day intravenous injections of KS-IL2 for 5 consecutive days). Serum was evaluated for the presence of mouse anti-human antibodies (MAHA) by ELISA. We found a rapid elevation of anti-human IgG antibodies in mouse serum after 4 days of treatment with huKS-IL2 fusion protein.

Because of the potential clinical importance of the KS-IL2 fusion protein, pharmacokinetic studies were conducted using BALB/C mice. After injection the fusion protein was detected in mouse serum using ELISA recognizing the human immunoglobulin component of fusion protein. A very different clearance pattern was found when previously injected animals were analyzed, indicated that the MAHA response was able to neutralize the KS-IL2 in previously treated animals. These data would be relevant to *in vivo* protocols designed to provide enhanced antitumor efficacy through the prolonged use or repeated cycles of treatment proposed for the huKS-IL2 molecule.

Our ultimate goal was to determine how this molecule is likely to be effective in treating cancer patients in clinical settings and which treatment regimen will be most effective. Unfortunately huKS-IL2 was not yet available for clinical testing. At the same time, hu14.18-IL2 fusion protein, from the same family of molecules but of different specificity became available for clinical studies at the UW-CCC. Thanks to our collaborators, we had the access to patients' samples from an initial Phase I clinical trial of this novel immunocytokine, delivered as a single agent therapy to the patients with GD-2 positive tumors. We decided to take advantage of this rare opportunity to take our research study to the next level by using these clinical specimens for model-building and assay development purposes, and to confirm by these experiments our previous findings with huKS-IL2 fusion protein in laboratory animals setting.

We evaluated samples from patients who received 4 hour infusions of the fusion protein on days 1,2 and 3 every 28 days (one course of treatment). Decreased lymphocyte count during the treatment was observed from day 1 through day 4 and then increased significantly starting from day 5 and continuing to the end of the first course of treatment. These findings were similar to our previous experience with clinical trials involving IL-2 administration (18). Flow cytometric technique was used also for analysis of surface markers on these cells. Significant expansion of CD16 and CD56 cells was observed on day 8 of treatment in comparison to day 1. This suggested stimulatory influence of fusion protein administration on immune cells of treated patients (19)

We adapted our previously developed for KS-IL2 fusion protein ELISA method for evaluation of serum specimens from patients subjected to ch14.18-IL2 fusion protein therapy. By this sensitive assay we are able to measure fusion protein concentration as low as 0.2 ng/ml with accuracy >95% (15). In the evaluated specimens the peak serum level of fusion protein was

observed immediately after the end of infusion (4 hrs), and fusion protein was detectable through 16 hrs after infusion started. The concentration of fusion protein in serum was dependent on the dose administered to the patients.

Similarly to our previous experiments *in vitro* with KS-IL2 fusion protein, we evaluated serum specimens obtained from patients subjected to fusion protein therapy to assess ability of fusion protein remaining in these serum samples to bind to GD-2 positive cells *in vitro*. Binding of fusion protein to the cell surface was detected by flow cytometry using antibody recognizing human IL-2. These experiments indicated that fusion protein present in these specimens retains its binding ability to GD-2 disialoganglioside expressed on the cell surface of control cell lines, and that the integrity of the fusion protein molecule is intact. These results correlated well with our previous findings with huKS-IL2 on mouse cell lines.

By functional testing of these patients' specimens as a source of IL-2 for an IL-2 responsive cell line Tf-1β (20), we proved that cytokine component of fusion protein present in circulation is active. This ability to induce cell proliferation was detected immediately after starting of the fusion protein infusion and remained for several hours after the end of fusion protein administration.

Additionally, specimens obtained at the end of hu14.18-IL2 infusion, representing the highest level of fusion protein were able to facilitate significantly higher ADCC *in vitro*, than specimens obtained prior to the infusion, as tested in a ^{51}Cr release assay (21) with effector cells from normal donor and GD-2 positive LAN-5 cells used as targets. This result indicated that serum from patients during fusion protein therapy can serve as a source of antibody for ADCC and further that the fusion protein present in patient circulation elicits ADCC on normal effector cells.

Moreover, PBMCs obtained from patients completing 3 infusions of hu14.18-IL2 fusion protein were more potent in facilitating ADCC *in vitro*, in comparison to the pretreatment PBMCs, on LAN-5 targets in the presence of fusion protein in the assay medium. This result indicates activation of patient's immune cells by fusion protein therapy. After fusion protein administration, sIL-2Rα levels measured in serum, increased in both courses of treatment, which is another demonstration of immune activation.

KEY RESEARCH ACCOMPLISHMENTS :

- generation and analysis of series of novel expression vectors for CC49-IL2 fusion protein, based on recombinant replication defective retroviruses, including CMV promoter
- applying the new gene delivery technology (PGMT) for the transfection of cell lines *in vitro* and skin transfection of the laboratory animals *in vivo*
- establishing immunotherapy model in which antibody-reactive tumors were engrafted into syngeneic immunocompetent murine hosts
- confirming that fusion proteins composed of tumor-specific antibody (KS or 14.18) and IL-2 are able to bind to the appropriate tumor cells (KSA or GD2 positive) where the IL-2 is recognized by anti-IL2 antibody
- these fusion proteins stimulate IL-2 dependent cell lines *(in vitro)* and mediate specific destruction of tumor targets by effector cells (obtained from patients previously subjected to IL-2 therapy)

- IL-2 receptor complex is involved in the enhanced killing of target cells induced by fusion proteins, and this interaction can be blocked by antibody against IL-2R beta subunit

- specific ELISAs have been established which allow sensitive detection of the intact fusion protein molecule, its immunoglobulin component and its cytokine component in mouse serum and in human serum

REPORTABLE OUTCOMES : (publications and meeting presentations)

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10. Ostendorf AM, Albertini MR, Gan J, Hank JA, Gillies SD, Sondel PM. Development of an assay to detect anti-hu14.18-IL2 response following in vivo therapy with anti-GD2 fusion protein, hu 14.18-IL2. American Association for Cancer Research Annual Meeting, San Francisco, 2000. Abstract.
11. Gan J, Imboden M, Surfus J, Hank JA, Sondel PM. Preclinical development of antibody-cytokine fusion protein for cancer immunotherapy. Era of Hope - DOD Breast Cancer Research Program Meeting, Atlanta, 2000. Abstract.

CONCLUSIONS :

Particle-mediated gene transfer (PMGT) has been shown to be an effective method of delivering genes that can recruit host immune effector cells to tumor sites to exert anti-tumor effects. Cytokine genes have primarily been used to engender such responses (22). We attempted to extend these observations using a gene encoding for antibody-cytokine fusion protein. Though we were able to generate readily detectable expression of the antibody-cytokine fusion protein at the delivery site and in the serum of laboratory animals, we were unable to show specific targeting or retention of the fusion protein in antigen positive tumor. Furthermore, despite our ability to create CC49 reactive murine cells, we could not show a statistically significant anti-tumor effect in syngeneic tumor engrafted animals following PMGT.

Despite of these difficulties in this setting, substantial progress has been made in our studies of antibody-IL2 fusion proteins potentially useful as a protein reagents for treatment of human breast cancer. We demonstrated that the huKS-IL2 fusion protein can specifically bind to human breast cancer cells and deliver IL-2 to their surface. It can activate IL-2 responsive cells, and induce tumor specific ADCC. We have adapted murine models that allow *in vivo* testing of KS-IL2 as a means for destroying KSA (+) tumor cells *in vivo*. Additionally, using clinical specimens from ongoing separate clinical trial with a similar molecule, hu14.18-IL2, we were able to confirm our previous findings with KS-IL2 *in vitro*. The preliminary data showed that is possible to safely administer fusion protein to the cancer patients, this fusion protein remains stable in the circulation for several hours without loosing its functional activity and induces immune activation. We have developed sensitive assays to detect this molecule in obtained specimens. The most recent work is based on GD-2 expressing tumors which represent a minority of human cancers. However, the principles which will emerge from this series of studies is likely to have broad applicability to antibody-based targeting of human cancers in general. Findings from studies of the hu14.18-IL2 fusion protein administration as a single agent, and in the near future in conjunction with other molecules, can readily be applied to other tumor models using immunocytokines involving antibodies directed at specific tumor targets. Further, *in vitro* and preclinical studies, as well as clinical trials will elucidate some of the mechanisms involved in enhancing immune effectors against tumor cells, and these principles, too, will be broadly applicable. The future clinical trials designed based on the results of pre-clinical and *in vitro* experiments will provide a model for bringing novel immunocytokines to the clinic, and hopefully will improve effectiveness of the treatment of human cancers.

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APPENDICES :

List of abbreviations :

ADCC	Antibody Dependent Cellular Cytotoxicity
ELISA	Enzyme-Linked Immunosorbent Assay
EpCAM	Epithelial Cell Adhesion Molecule
FP	Fusion Protein
GD-2	Disialoganglioside
hu14.18-IL2	Humanized 14.18 antibody/interleukin-2 fusion protein
IC	Immunocytokine
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-2R	Interleukin-2 receptor
LAN-5	Human neuroblastoma cell line
M-21	Human melanoma cell line
mAb	Monoclonal Antibody
NK	Natural Killer Cell
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
sIL-2Rα	Soluble Interleukin-2 receptor Alpha Subunit
UW-CCC	University of Wisconsin Comprehensive Cancer Center

Fig. 1. Activity of Expression Constructs

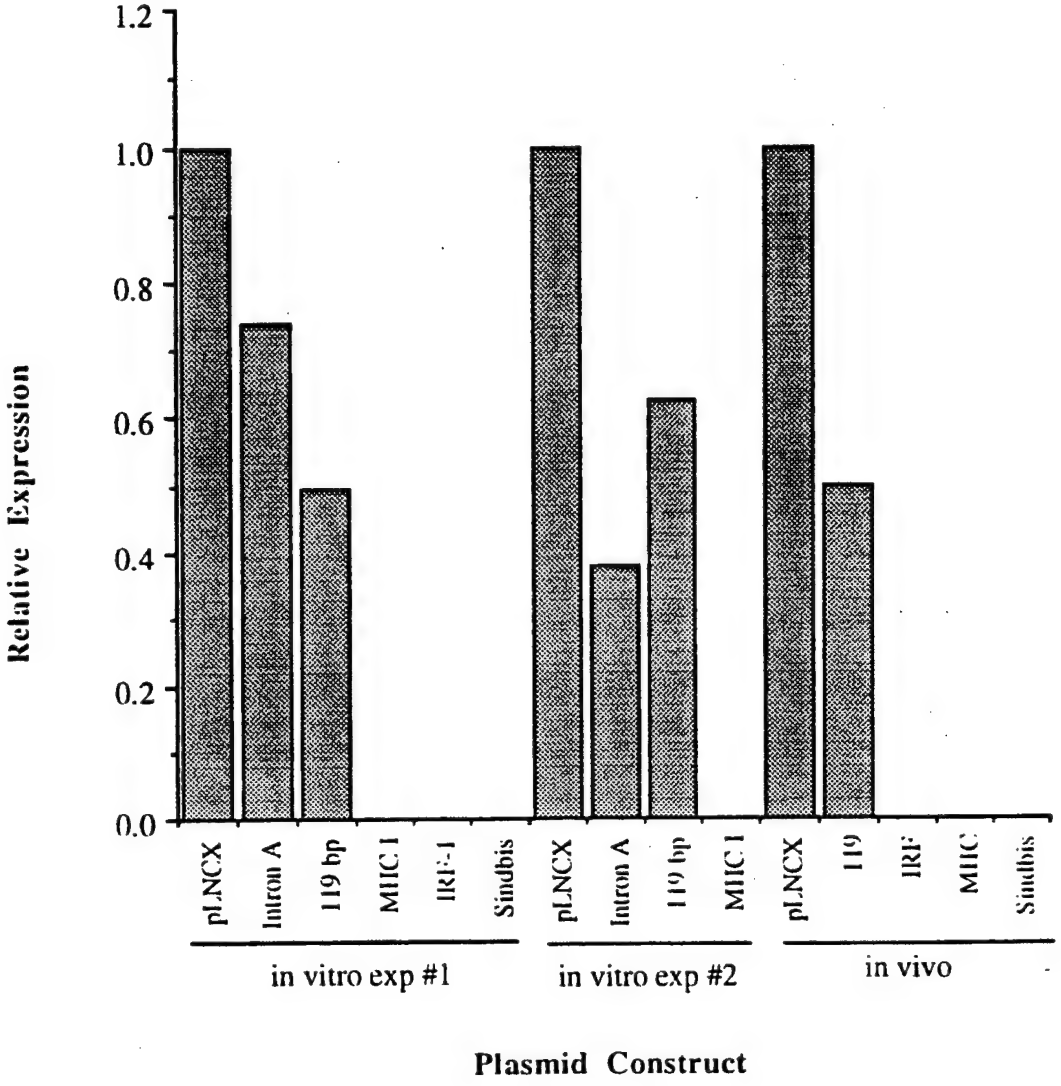


Figure 1. Relative expression levels of various plasmid constructs, all driving production of the CC49-IL2 FP, are shown, with the expression of the pLNCX parental vector set as 1.0. The salient features of the different vectors are described in the text. Whether the experiments were done in vitro, assaying production in B16 melanoma cells, or in vivo, assaying production in mouse skin, is indicated.

Table I Retention of CC49-IL2 FP by Different Tissue Sites in Nude Mice

	Neutral (TAG 72-)	M21 (TAG 72-)	LS174T (TAG 72+)
24 hr.,A	113.90	48.10	24.00
24 hr..B	96.50	79.00	34.00
96 hr..A	0.10	0.07	<0.05
96 hr..B	0.11	0.28	0.12

Data from each animal,sacrificed at different time points following PMGT with the pLNC-CC49 IL2 expression vector, are presented in ng/ml of FP. TAG-72 antigen status was defined by reactivity with the CC49 antibody. Approximately 100 mg of tissue are disrupted per 0.5 ml lysis buffer.

Table II. Targeting of Systemic CC49-IL2 FP to Antigen Positive Sites

Site:	Animal 1	Animal 2	Animal 3	Animal 4
Transfected (FP secreting) Tumor	321.0	1120.0	1093.0	249.0
Serum	4.7	6.7	49.8	2.8
Untransfected Tumor	0.1	0.3	0.6	<0.05
Neutral	0.6	<0.05	0.3	<0.05

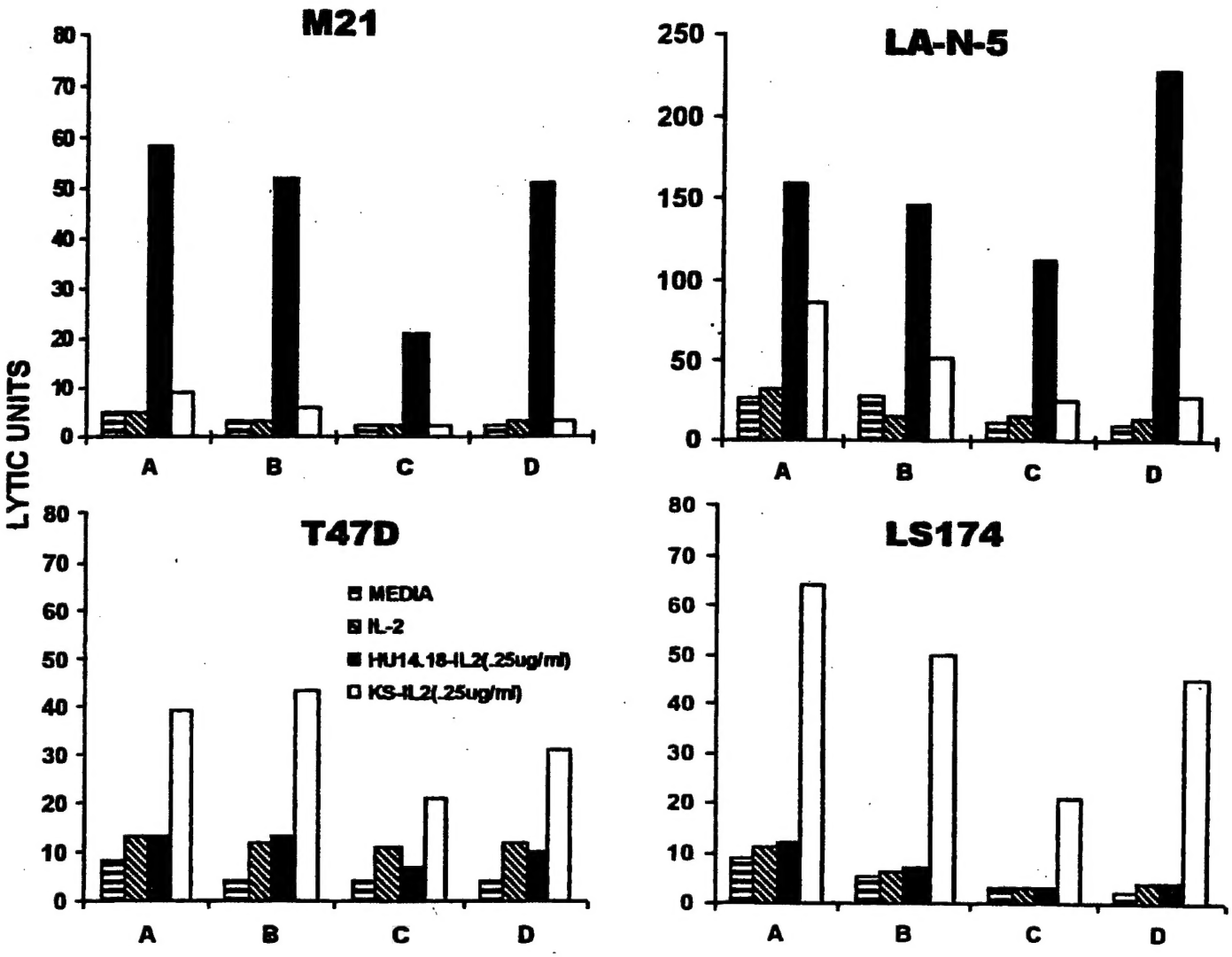
Data are presented in ng/ml of FP. Nude mice were engrafted with antigen positive LS174T cells transfected or not with an expression vector producing the CC49-IL2 FP. Tumors were harvested at a diameter of 3-5 mm, and processed in 0.5 ml of lysis buffer.

Table III. CC49-Reactive Antigen Expression in Transfected TSA Murine Mammary Adenocarcinoma Cells

	Primary Antibody	Gated Population	% Gated Cells	Mean Fluorescence Intensity
Untransfected TSA	MOPC 21	All	100	4.4
		M1	0.4	28.4
	CC49	All	100	6.4
		M1	2.8	45.3
Transfected TSA	MOPC 21	All	100	7.9
		M1	4.6	39.5
	CC49	All	100	42.62
		M1	20.5	176.4

TSA Cells transfected or not with a MUC-1 expression plasmid were analyzed for reactivity with the CC49 antibody by flow cytometry. MOPC-21 is used as a non-reactive isotype control. "M1" corresponds to a marker defining a region of more brightly staining cells, and is established at the same coordinates in all experimental samples.

Specificity of KS-IL2 & hu14.18-IL2 in Mediating ADCC



Freshly obtained peripheral blood mononuclear cells from four healthy control donors (A,B,C,D) were used as effector cells in a 4 hour chromium release assay with M21 a human melanoma target, LA-N-5 a human neuroblastoma target, T47D a human breast carcinoma target and LS174 a human colon carcinoma target. The cytotoxicity mediated by serial dilutions of effector cells was measured on these target cells in media alone, in IL-2 (100U/ml), in KS-IL2 fusion protein (0.25µg/ml) and in hu14.18-IL2 (0.25µg/ml). The data, shown in lytic units, indicate that the KS-IL2 fusion protein specifically binds to the T47D and LS174 tumor targets and interacts with the effector cells to facilitate ADCC. Furthermore, the KS-IL2 fusion protein does not bind to the M21 melanoma or the LA-N-5 neuroblastoma cell line. The hu14.18-IL2 fusion protein binds to the M21 and LA-N-5 tumor target and results in good ADCC. The hu14.18-IL2 fusion protein does not bind to the T47D or the LS174 targets nor induce ADCC of them, thereby demonstrating specificity.

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Specific Enzyme-Linked Immunosorbent Assays for Quantitation of Antibody-Cytokine Fusion Proteins

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Preliminary testing has shown in vitro and in vivo that antitumor activity can be obtained with fusion proteins linking tumor-reactive monoclonal antibodies to cytokines, such as granulocyte-macrophage colony-stimulating factor or interleukin 2 (IL-2). Preclinical and clinical testing of these reagents requires their in vitro and in vivo quantitation and pharmacokinetic evaluation. We have focused on the detection of a fusion protein which links one human IL-2 molecule to the carboxy terminus of each heavy chain of the tumor-reactive human-mouse chimeric anti-GD2 antibody, ch14.18. We have developed enzyme-linked immunosorbent assays (ELISAs) to evaluate intact tumor-reactive fusion proteins. By these ELISAs we can reliably measure nanogram quantities of intact ch14.18-IL-2 fusion protein and distinguish the intact protein from its components (ch14.18 and IL-2) in buffer, mouse serum, and human serum with specificity and reproducibility. The measurement of intact ch14.18-IL-2 fusion protein is not confounded by free IL-2 or free ch14.18 when 100 ng or less of total immunoglobulin per ml is used during the assay procedure. Our results indicate that these ELISAs are suitable for preclinical and clinical testing and with slight modifications are applicable to the analysis of a variety of other fusion proteins.

Through molecular engineering, proteins can be altered to enhance their bioactivities. Fusion proteins, designed to combine antibodies with cytokines (2, 3, 5, 6, 13, 16, 17, 19), antibodies with cytokine receptors (1), or cytokines with toxins (8), are currently being evaluated in preclinical and clinical studies (18). The ch14.18-interleukin 2 (IL-2) and hu14.18-IL-2 proteins are two such engineered, antitumor antibody-cytokine fusion proteins (3). Human recombinant IL-2 has been linked to the anti-GD2 human-mouse chimeric or humanized forms of the 14.18 antibody (ch14.18 or hu14.18) at the carboxy terminus of the immunoglobulin heavy chain. The ch14.18-IL-2 fusion protein was shown to enhance in vitro killing of autologous GD2-positive human melanoma cells by a tumor-infiltrating lymphocyte cell line (3). In vivo, ch14.18-IL-2 markedly inhibited the growth of established hepatic metastases in severe combined immunodeficient (SCID) mice, previously reconstituted with human lymphokine-activated killer cells (15), and in immunocompetent mice bearing syngeneic GD2+ tumors (10). Increasing interest in the use of antibody-cytokine fusion proteins such as these in the treatment of malignant diseases warrants a systematic approach for quantifying and assessing their immunopharmacological effects in preclinical and clinical trials.

Many of the standard methods of protein quantitation lack specificity. For example, spectrophotometric assays are confounded by other proteins in the serum (Bradford, Lowry, or bicinchoninic acid protein assay systems), and enzyme-linked immunosorbent assays (ELISAs) quantitating immunoglobulin G (IgG) are unable to distinguish the intact fusion protein from the parent immunoglobulin. The assays described in this report specifically quantitate and distinguish the intact fusion protein from its breakdown or composite products, by utilizing capture reagents directed against one functional group and detection ligands which combine with the other active moiety. The potential use of bioengineered fusion proteins in vivo necessitates the development of assays which accurately determine the quantity of intact fusion protein. The assays presented here should be useful for both in vitro and in vivo evaluations of a wide variety of fusion proteins used in both preclinical and clinical testing.

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MATERIALS AND METHODS

Immunologic reagents. (i) Antibody-IL-2 fusion proteins. Antibody-cytokine fusion proteins used in this study include ch14.18-IL-2 and hu14.18-IL-2 (obtained from Toby Hecht of the National Cancer Institute [NCI], Frederick, Md.), CC49-IL-2 (obtained from Jeff Schlom of the NCI), and KS1/4-IL-2 (Lexigen Pharmaceuticals). The ch14.18-IL-2 fusion protein contains a mouse-human chimeric IgG1 with an anti-GD2 recognition domain and a human IL-2 molecule at the carboxy terminus of each heavy chain (3). The purification of the ch14.18-IL-2 fusion protein used in these studies was performed at the Monoclonal Antibody and Recombinant Protein facilities (NCI), and two independently purified batches (lot numbers 1 and 31403) were used as indicated. Stock concentrations of these two lots, based on ELISAs of their IgG content, were 1.15 and 0.4 mg/ml, respectively. hu14.18-IL-2 was obtained at a concentration of 1.0 mg/ml and stored until use. The CC49-IL-2 fusion protein is a single-chain antibody-cytokine fusion protein. It contains the antigen recognition domain from the murine monoclonal antibody (Mab) CC49, a human IgG1 heavy chain, and human IL-2 (22). The CC49-IL-2 protein was purified from culture supernatants of expressing cells (22, 14) and maintained as a stock solution at a concentration of 200 μg/ml. KS1/4-IL-2 is a humanized antibody-IL-2 fusion protein which is similar in structure to the hu14.18-IL-2 molecule but uses the humanized form of the mouse KS1/4 pan-carcinoma antibody (26). The stock of high pressure liquid chromatography-purified KS1/4-IL-2 determined by spectrophotometric measurement was at a concentration of 1 mg/ml. Concentrations of immunoglobulins were verified by using an ELISA for IgG content. The purity and structural integrity of the proteins were verified by electrophoretic and Western blot analyses. All fusion proteins were stored at −80°C until use.

(ii) Anti-idiotype antibodies. The anti-idiotype antibodies used include Mab 1A7 (provided by K. Foon and M. Chatterjee of the University of Kentucky, Lexington, and Titan Pharmaceuticals, Inc.), a murine IgG1, which recognizes the hypervariable region of the ch14.18 Mab (20), and A.I. 49-3, a monoclonal anti-idiotype antibody against the mouse CC49 antibody obtained from J. Schlom (NCI). These were stored at −80°C until use.

(iii) Other immunoreagents. ch14.18 is a mouse-human Mab which recognizes the GD2 antigen found on melanomas and neuroblastomas. The antibody was

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PRECLINICAL DEVELOPMENT OF ANTIBODY-CYTOKINE FUSION PROTEIN FOR CANCER IMMUNOTHERAPY

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Our research team has pursued preclinical and clinical investigations combining the administration of IL-2 with tumor-reactive monoclonal antibodies. This cancer treatment approach unfortunately has some limitations connected with toxicity of IL-2 as well as generation of the immune response directed to antibody molecule. In recent years, using recombinant DNA technology it has been possible to create the new molecules called fusion proteins or immunocytokines. These molecules consist of a modified (chimeric or humanized) tumor-specific antibody, which heavy chains are covalently linked to a cytokine. In experiments with KS-IL2 fusion protein, recognizing the KSA antigen expressed on a broad range of human carcinomas, including breast cancer, we were able to demonstrate that in laboratory settings this immunocytokine can specifically bind to the KSA-positive human cancer cell lines (including breast, colon , lung) and deliver a cytokine to their surface. Also this molecules can stimulate IL-2 dependent cell lines (in vitro) as well as activate human immune cells and induce tumor specific ADCC. We have designed a specific ELISAs to monitor the presence of the intact fusion protein molecule as well as its components in animal and human circulation. One of these immunocytokines of different specificity (hu 14.18-IL2) is currently in clinical testing in our institution, given as a single agent therapy to the patients with GD-2 positive tumors. We are now studying a pharmacokinetics of this molecule in patients serum and parameters of immune stimulation during this treatment. We found that this molecule is detectable for several hours in patients serum and is retaining its activity in vitro. Also we found the increasing levels of IL-2Rα which is considered as a marker of immune activation. Studies are underway to assess the effects of this treatment and its safety at doses that induce striking immunological activation. Findings from these preliminary studies will provide a baseline for clinical testing of other novel immunocytokines targeting human cancers. It will help to design the future clinical trials involving administration of fusion proteins as single agents or in combination with other immunostymulants and hopefully will improve effectiveness of the treatment of human cancer.

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